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Cloning and Sequencing of the *Escherichia coli panB* Gene, Which Encodes Ketopantoate Hydroxymethyltransferase, and Overexpression of the Enzyme

CAROL E. JONES,[1,2] JUDITH M. BROOK,[3] DAVID BUCK,[3] CHRIS ABELL,[2] AND ALISON G. SMITH[1]*

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA,[1] University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW,[2] and Schering Agrochemicals Ltd, Chesterford Park Research Station, Saffron Walden, Essex, CB10 1XL,[3] England

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The *panB* gene from *Escherichia coli*, encoding the first enzyme of the pantothenate biosynthesis pathway, ketopantoate hydroxymethyltransferase (KPHMT), has been isolated by functional complementation of a *panB* mutant strain with an *E. coli* genomic library. The gene is 792 bp long, encoding a protein of 264 amino acids with a predicted M_r of 28,179. The identity of the gene product as ketopantoate hydroxymethyltransferase was confirmed by purification of the enzyme protein, which was overexpressed approximately 50-fold in the mutant harboring the gene on a high-copy-number plasmid. The N-terminal amino acid sequence of the purified protein was found to be identical to that predicted from the gene sequence, as was its mass, determined by electrospray mass spectrometry. Upstream of the *panB* gene is an incomplete open reading frame encoding a protein of 220 amino acids, which shares sequence similarity to fimbrial precursor proteins from other bacteria. Northern (RNA) analysis showed that the *panB* gene is likely to be cotranscribed with at least one other gene but that this is not the putative fimbrial protein, since no transcripts for this gene could be detected.

Pantothenic acid is the precursor of coenzyme A. It is synthesized by microorganisms and plants but not mammals, which require it as part of their diet. The three-step pathway to pantothenic acid has been studied in microorganisms (4, 15, 16) and is shown in Fig. 1. The first committed step is the formation of ketopantoate from α-ketoisovalerate, catalyzed by the enzyme ketopantoate hydroxymethyltransferase (KPHMT; 5,10-methylene-tetrahydrofolate:α-ketoisovalerate hydroxymethyltransferase, EC 2.1.2.11). KPHMT has been purified to homogeneity from wild-type *Escherichia coli* by Teller et al. (28).

Pantothenate-requiring *E. coli* mutants were first generated in the early 1950s by UV irradiation (15, 16) and indeed were instrumental in establishing the precise biochemical pathway of pantothenate synthesis. Thus, the *panB* mutant, which is completely lacking in KPHMT, requires ketopantoic acid, pantoic acid, or pantothenic acid for growth (7). With conjugational crosses and phage transduction, the *panB* gene was shown to be closely linked to the genes for two other enzymes, pantothenate synthetase and aspartate 1-decarboxylase (designated *panC* and *panD*, respectively), and they have been mapped on the *E. coli* chromosome at 3.1 min (6). In order to study pantothenate synthesis in *E. coli* more thoroughly, we decided to isolate the *panB* gene and to characterize its gene product. In this paper we describe the isolation and characterization of the gene and the overexpression and purification of the encoded enzyme. This is the first reported sequence of a gene on the pantothenate pathway.

* Corresponding author. Electronic mail address: AS25@mbl.bio.cam.ac.uk.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* Hfr3000 YA139, a *panB* mutant derivative of *E. coli* K-12 (6), was used to isolate the *panB* gene by functional complementation. The mutant was grown on GB1 minimal medium (13.6 g of KH_2PO_4 [pH 7.0], 2 g of $(NH_4)_2SO_4$, 4 g of glucose, 0.25 g of $MgSO_4$, 0.25 μg of $FeSO_4$, 5 mg of vitamin B_1, and 5 mg of pantothenic acid per liter. *E. coli* K-12 was used for the generation of an *E. coli* genomic library and for enzyme assays. The *E. coli* strain XL1-Blue (Stratagene) was used for the propagation of plasmids. The vector pBluescript M13 (pKS^-) was from Stratagene and was used for all cloning experiments.

DNA manipulations. All restriction enzymes were purchased from Boehringer, and digestions were performed under the conditions recommended by the manufacturer. T4 DNA ligase and calf intestinal phosphatase were also from Boehringer, and plasmid manipulations were carried out by the method of Sambrook et al. (24). The isolation of DNA fragments for subcloning after electrophoresis in agarose (Bio-Rad) was carried out as previously described (24), as were transformations in the presence of calcium chloride. Alkaline minipreparations of DNA were prepared by the method Birnboim and Doly (2). Cesium chloride purifications of DNA were carried out by the method of Sambrook et al. (24).

Generation of unidirectional exonuclease III deletions. Exonuclease III deletions of the plasmid pCEJ01 were performed with exonuclease III and mung bean nuclease purchased from Stratagene in the form of a kit. The reactions were carried out by a method described in the pBluescript Exo/Mung DNA sequencing system instruction manual.

DNA sequence analysis. All the components for DNA sequence analysis were purchased in the form of a kit from United States Biochemical Corp.; Sequenase 2.0 was used for sequencing, and reactions were carried out by meth-

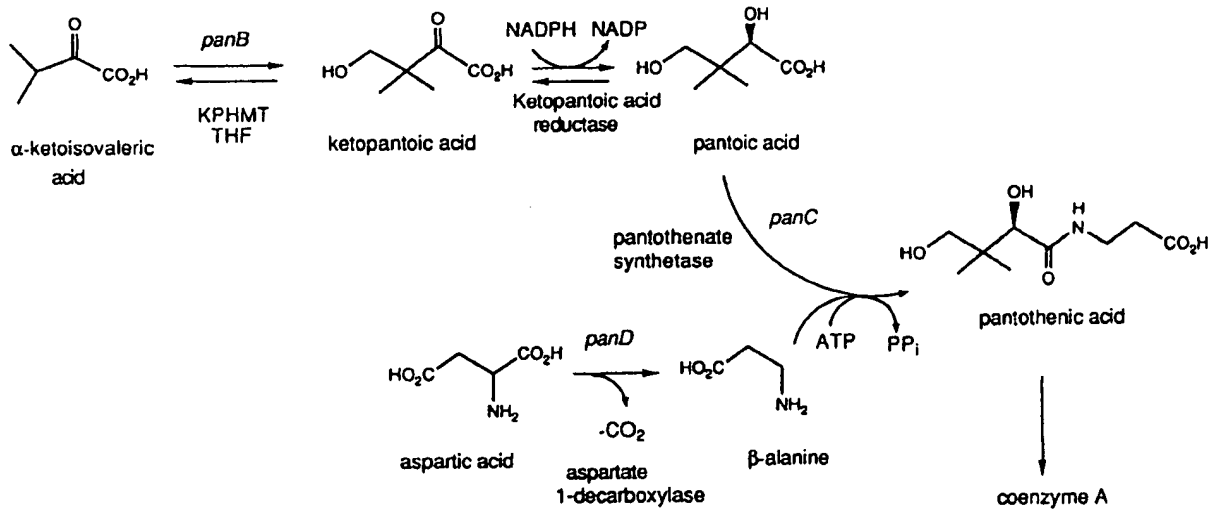

FIG. 1. Pantothenic acid biosynthetic pathway.

ods recommended by the manufacturer. [α-^{35}S]dATP (3,000 Ci/mmol) was purchased from Amersham International. Electrophoresis was on buffer gradient gels (1). Analysis of DNA and amino acid sequences were performed with Staden programs (26) and Genetics Computer Group analysis software package version 7.0 (8). *panB*-specific oligonucleotide primers used to sequence part of the fragment were obtained from the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge.

Northern (RNA) analysis. Total cellular RNA was prepared from log-phase cultures of *E. coli* strains by the method of Duncan and Coggins (9) and purified on cesium chloride gradients to remove contaminating DNA. It was glyoxylated and electrophoresed on 1.2% agarose gels before being blotted onto Gene Screen Plus nylon filters essentially as described by Sambrook et al. (24). Blots were prehybridized for 3 h in 1% sodium dodecyl sulfate (SDS)–1 M NaCl–10% dextran sulfate at 60°C and then hybridized at the same temperature overnight with radiolabelled probe in fresh hybridization buffer. Blots were washed twice for 30 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS at 60°C and then autoradiographed at −70°C for 1 to 7 days. Probes were fragments of DNA excised from agarose gels and radiolabelled with [α-^{32}P] dCTP (3,000 Ci/mmol; Amersham International) by the random oligonucleotide method of Feinberg and Vogelstein (11).

PAGE in the presence of SDS. Polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of SDS by the method of Laemmli (14) with a 15% running gel and a 5% stacking gel.

Enzyme assays. Enzyme assays for KPHMT were performed in the reverse direction with ketopantoate as a substrate and by measuring the amount of formaldehyde produced with the Nash reagent, as described by Teller et al. (28). Ketopantolactone, used to prepare the substrate ketopantoate for the enzyme assay, was synthesized by the method of Ojima et al. (18).

Purification of KPHMT. The purification procedure was based on that described by Teller et al. (28) and used identical buffers and the same method for crude cell lysate preparation. However, the column matrices used were slightly different, and the chromatography was performed with a Pharmacia fast-protein liquid chromatography system. A 750-ml culture of the *E. coli panB* mutant containing plasmid pCEJ01 was grown overnight at 37°C, and cells (7 to 15 g [wet weight]) were harvested by centrifugation at 8,000 × *g* for 15 min. They were resuspended in 14 ml of buffer A (50 mM KH_2PO_4 [pH 6.8], 1 mM EDTA, 10 mM 2-mercaptoethanol) and lysed by sonication. After removal of debris by centrifugation at 15,000 × *g* for 30 min, the crude lysate was applied to a DEAE-Sepharose anion-exchange column (28 by 2.6 cm; Pharmacia) equilibrated in buffer A and eluted with a salt gradient (0.2 to 0.5 M KCl). The fractions containing KPHMT activity were concentrated by bringing to 70% ammonium sulfate saturation. The pellet was recovered by centrifugation at 15,000 × *g* for 15 min and resuspended in 400 μl of 100 mM KH_2PO_4 (pH 6.8)–1 mM EDTA–10 mM 2-mercaptoethanol and dialyzed against the same buffer. The sample was applied to a Superose 12 HR10/30 column (Pharmacia) and eluted in the same buffer. KPHMT-containing fractions were heated to 80°C for 5 min, and denatured proteins were removed by centrifugation. KPHMT remained in the supernatant. Protein concentration was determined by the method of Bradford (3).

N-terminal amino acid determination. The N-terminal amino acid sequence of the purified KPHMT was determined by the Protein and Nucleic Acid Chemistry Facility in the Department of Biochemistry, University of Cambridge.

Electrospray mass spectrometry. Electrospray mass spectrometry was performed on a VG BioQ mass spectrometer. Samples containing approximately 200 pmol of protein were applied in 10 μl of MeOH-H_2O-acetic acid (50:50:1) at a flow rate of 4 μl/min.

Nucleotide sequence accession number. The sequence of the 1.7-kb *Eco*RI-*Sal*I fragment containing the *panB* gene has been deposited at EMBL under accession number X65538.

TABLE 1. Assays for KPHMT activity in crude lysates of *E. coli* wild type, *panB* mutant, and *panB* mutant harboring various plasmids

E. coli strain and plasmid	Sp act (μmol min^{-1} mg^{-1})	Relative sp act
K-12	0.007	1
Hfr3000 YA139	0.0	0
Hfr3000 YA139/pSAL38	0.666	95
Hfr3000 YA139/pCEJ01	0.348	50
Hfr3000 YA139/pCEJ02	0.162	23
Hfr3000 YA139/pCEJ03	0.0	0
Hfr3000 YA139/pCEJ04	0.0	0

RESULTS

Isolation of the *E. coli panB* gene. The *E. coli panB* gene was isolated by functional complementation of *E. coli* HFr3000 YA139, which carries the *panB* mutation and lacks KPHMT activity (6). Cells of the mutant were made competent, transformed with an *E. coli* *Eco*RI genomic plasmid library, and plated out onto minimal media containing ampicillin but no pantothenic acid. Twenty-three colonies (of a total of 1,100 transformants) grew and so presumably had restored KPHMT activity. Six were taken for further analysis, and each was shown to contain a plasmid with an insert of 2.5 kbp. Crude lysates of *E. coli* Hfr3000 YA139 harboring the clones were assayed for KPHMT activity by the method described by Teller et al. (28) and showed levels of KPHMT activity 20- to 100-fold higher than wild-type *E. coli* K-12 (Table 1). One of the isolated plasmids, designated pSAL38, was chosen for further subcloning and sequencing.

Restriction mapping of pSAL38. The restriction map of pSAL38 is shown in Fig. 2. Four subclones (pCEJ01 to pCEJ04) were prepared on the basis of the restriction map and used to retransform *E. coli* Hfr3000 YA139. Only clones pCEJ01 and pCEJ02 were able to complement the *panB* mutation, allowing the mutant to grow in the absence of pantothenate. Enzyme assays for KPHMT on crude cell lysates with all four clones confirmed the functional complementation results (Table 1). The smallest clone, pCEJ01, contained a 1.7-kb *Eco*RI-*Sal*I insert. This was sequenced in both directions by the Sanger dideoxy chain termination method (25).

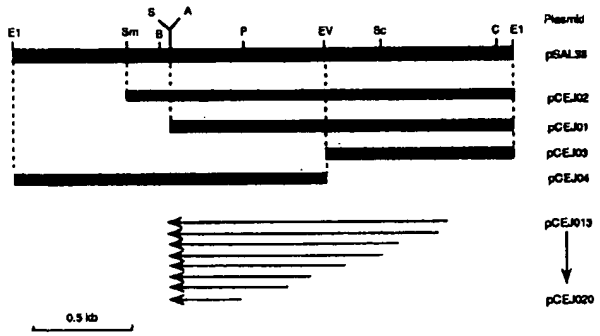

FIG. 2. Restriction map and subcloning strategy for plasmid pSAL38. pCEJ01 to pCEJ04 were subcloned from pSAL38 into pBluescript. The diagrams also shows a series of nested deletion clones (pCEJ013 to pCEJ020) generated by exonuclease III deletion from the *Eco*RI end of plasmid pCEJ01. E1, *Eco*RI; *Sm*, *Sma*I; B, *Bgl*I; S, *Sal*I; A, *Acc*I; P, *Pvu*II; EV, *Eco*RV; Sc, *Sca*I; C, *Cla*I.

Nucleotide sequence of the *panB* gene. The nucleotide sequence of the 1.7-kb *Eco*RI-*Sal*I fragment containing the *panB* gene is shown in Fig. 3. Analysis of the sequence with the STADEN nucleotide interpretation program (version 4.1) revealed two open reading frames (ORFs) transcribed from the same strand. All other reading frames had multiple stop codons. The first ORF, which is not complete, is 660 bp in length, and the second is 792 bp.

A series of eight nested deletions from the *Eco*RI site of the 1.7-kb insert of pCEJ01 (Fig. 2) were generated with exonuclease III as described in Materials and Methods. Transformation of Hfr3000 YA139 with these clones suggested that the *panB* gene lay within the second ORF of 792 bp, since deletions which interrupted this ORF did not complement the mutation. The orientations of the ORFs are such that the gene must be transcribed from its own promoter, as the promoters of the ampicillin resistance and *lacZ* genes in the vector would transcribe the other strand.

A putative ribosome binding site (AGGA, overlined on Figure 3) is situated 7 bp upstream of the ATG start codon of the *panB* gene. A putative promoter sequence (boxed in Fig. 3) which starts 35 bp 5′ to the initiating ATG codon was identified by its similarity to the consensus −35 and −10 sequences (13), although clearly its authenticity cannot be confirmed without functional analysis.

Sequence comparison of the first ORF with the PIR28 data base (11.6.1991) found that there were short stretches (30 to 80 amino acids) which had 30 to 50% sequence similarity with the sequences of fimbrial protein precursors of *Klebsiella pneumoniae*, *Serratia marcescens*, *Salmonella typhimurium*, *E. coli*, and *Haemophilis influenzae* (12, 17, 22, 29, 30), suggesting that this sequence codes for all or part of a fimbrial protein precursor.

Northern analysis. In order to determine whether either, or both, of the ORFs encoded by CEJ01 was part of an operon, Northern analysis was carried out. Figure 4 shows the result of probing RNA extracted from log-phase cultures of *E. coli* K-12 and Hfr3000 YA139 with probes internal to ORF1 and the *panB* gene (a 475-bp *Hinc*II-*Sma*I fragment and a 530-bp *Afl*III-*Pvu*II fragment, respectively). It is clear that in both wild-type and mutant strains, a major band of 1.9 kbp is detected with the *panB* gene-specific probe. Since the coding region of the *panB* gene is less than 800 bp, it is likely that it is cotranscribed with at least one other gene. However, this is not the putative fimbrial protein encoded by ORF1, since on an identical blot, no message is detectable for this gene (Fig. 4). Even when the blot was overexposed (for 7 rather than 2 days), no signal was observed, suggesting that ORF1 is not expressed in log-phase cultures growing in rich media, supporting previous observations (19). The fact that an identical message for *panB* is present in the mutant and wild-type cells, and in approximately the same amount, suggests that the defect carried by Hfr3000 YA139 is likely to be a point mutation in the coding region.

Purification of KPHMT overexpressed in Hfr3000 YA139 cells. In order to confirm the identity of the second ORF as the *panB* gene, the protein which it encodes was overexpressed and purified, and the N-terminal sequence was determined. The construct with clone pCEJ01 in Hfr3000 YA139 showed levels of KPHM 50-fold higher than those of the wild type in enzyme assays (Table 1). Furthermore, SDS-PAGE analysis of total soluble protein from the mutant harboring plasmid pCEJ01 showed the presence of a prominent band of 29,000 Da, which was absent in the mutant alone, or indeed from *E. coli* K-12 cells (Fig. 5). KPHMT was purified to homogeneity from the transformed cells by a

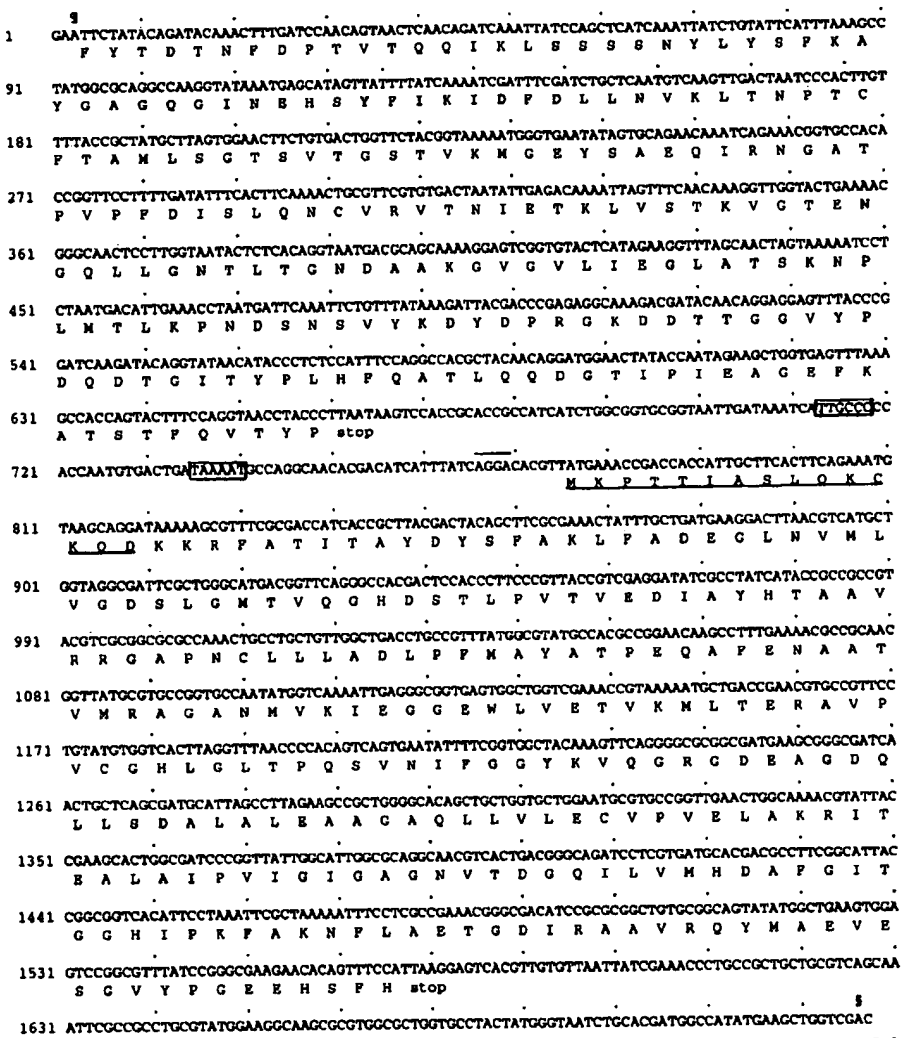

FIG. 3. Sequence of the 1.7-kbp fragment which complemented the *panB* mutation. The nucleotide sequence of the 1.7-kb *Eco*RI-*Sal*I insert in pCEJ01 is shown with the translated sequence of the putative fimbrial protein precursor (ORF1) and that of the translated *panB* gene shown below. The postulated ribosome binding site for *panB* is overlined, and the putative −10 and −35 promoter regions are boxed. The amino acid sequence determined experimentally from the purified protein is underlined. ¶, *Eco*RI site; §, *Sal*I site.

four-step procedure, detailed in Materials and Methods, which closely followed the original purification from wild-type *E. coli* (28). A crude cell lysate was subjected to anion-exchange chromatography, ammonium sulfate precipitation, gel filtration, and finally, a heat treatment. A 16-fold purification of KPHMT was achieved with a 57% recovery of activity (Table 2). The purification was followed by SDS-PAGE, and the heat-treated sample appeared as a single band of 29,000 Da in Coomassie (Fig. 5)- and silver-stained gels.

Analysis of purified KPHMT. N-terminal sequence analysis of the first 15 residues of purified KPHMT confirmed the translational start of the *panB* gene (underlined in Fig. 3) and the predicted protein sequence. The purified KPHMT sample was also subjected to electrospray mass spectrometry (10) (data not shown) and was shown to have a subunit molecular weight of 28,178 ± 5 (predicted molecular weight, 28,179). The native M_r was determined by gel filtration on a Superose 12 HR10/30 column to be 174,000, suggesting that the enzyme is a hexamer. The K_m value of KPHMT was determined for ketopantoate in the reverse enzyme-catalyzed reaction to be 0.15 mM. This compares well with that of 0.16 mM, determined by Powers and Snell (21).

DISCUSSION

In this paper we report the isolation of the *panB* gene encoding the pantothenate biosynthesis enzyme KPHMT from *E. coli* by functional complementation of an *E. coli* *panB* mutant (strain Hfr3000 YA139) deficient in KPHMT. After transformation of the mutant with a genomic library of *E. coli* DNA, a clone containing a 2.5-kb insert was isolated. Transformation of *E. coli* Hfr3000 YA139 with subclones of this initial fragment identified a smaller clone (pCEJ01) with

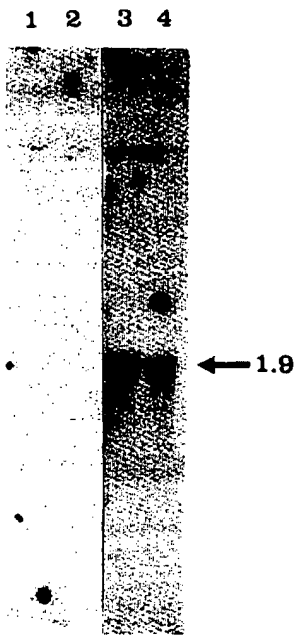

FIG. 4. Northern analysis of transcripts from the ORF1 and *panB* genes. Autoradiograph of a Northern blot of RNA extracted from *E. coli* K-12 (lanes 1 and 3) and Hfr3000 YA139 (lanes 2 and 4) probed with an internal fragment of ORF1 (lanes 1 and 2) or *panB* (lanes 3 and 4).

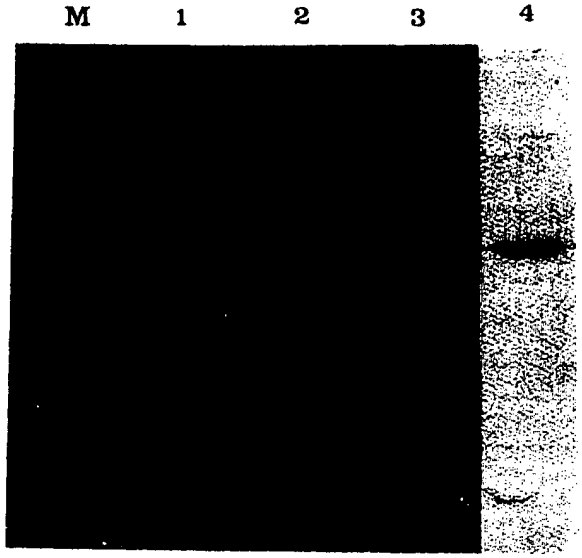

FIG. 5. SDS-PAGE analysis of overexpressed and purified KPHMT. Proteins were electrophoresed on a 15% polyacrylamide gel and visualized with Coomassie blue. Lanes: 1, 12.5 μg of protein from *E. coli* K-12; 2, 12.5 μg of protein from *E. coli* Hfr3000 YA139/pBluescript; 3, 12.5 μg of protein from *E. coli* Hfr3000 YA139/pCEJ01; 4, 0.6 μg of purified KPHMT; M, marker proteins (bovine serum albumin [66 kDa], ovalbumin [45 kDa], glyceraldehyde-3-phosphate dehydrogenase [36 kDa], carbonic anhydrase [29 kDa], trypsinogen [24 kDa], trypsinogen inhibitor [20 kDa], and α-lactalbumin [14 kDa]).

a 1.7-kb insert which included the entire *panB* gene. Sequence analysis revealed two ORFs of 660 and 792 bp. The smaller ORF shows sequence similarity to fimbrial protein precursor sequences (12, 17, 22, 29, 30). The second ORF of 792 nucleotides was confirmed as encoding KPHMT, by transformation of the *E. coli panB* mutant with a series of nested deletions of CEJ01; only those which did not interrupt this second ORF complemented the *panB* mutation.

When the amino acid sequence of *panB* was used to screen the available protein data bases (Swissprot, PIR, and GenBank) with the program FASTA, no similar proteins were identified. In contrast, when it was compared directly to enzymes known to carry out similar reactions, with tetrahydrofolate as cofactor, it was found to have some weak similarity to a region of serine hydroxymethyltransferases from several bacterial sources (Fig. 6). However, it is not possible to draw any conclusions about the functional significance of this, if any, until more is known about the binding of tetrahydrofolate to these enzymes.

KPHMT activity in *E. coli* Hfr3000 YA139 harboring plasmid pCEJ01 was 50-fold greater than levels in wild-type *E. coli* (Table 1), presumably because of the high copy number of the pBluescript vector. It was therefore relatively straightforward to purify KPHMT to homogeneity from the rescued mutant. The preparation yielded a single protein of 29,000 Da on SDS-PAGE (Fig. 5). N-terminal protein sequence analysis of this band confirmed the predicted translational start of the *panB* gene and the sequence of the first 15 residues. Significantly, the sequencing confirmed that the second residue is lysine, not tyrosine as had been reported by Powers and Snell (21).

The subunit molecular weight of KPHMT was determined by electrospray mass spectrometry to be 28,178 ± 5. This is in agreement with the predicted molecular weight of 28,179 and shows that there is no posttranslational modification of the enzyme. The native molecular mass of KPHMT was determined by gel filtration to be 174,000 Da, suggesting the enzyme is a hexamer. This is smaller than the values of 285,000 Da (gel filtration) and 255,000 Da (sedimentation equilibrium) reported by Teller et al. (28) for the enzyme purified from wild-type *E. coli*, but the yield they obtained for the enzyme was low, and their estimation of the subunit molecular weight (25,000) was also incorrect.

By genetic analysis, the *panB*, *panC*, and *panD* genes have been found to be closely clustered at 3.1 min of the *E. coli* K-12 genetic map (6, 7). In the same work, the clockwise order of the genes was found to be *panB panD panC* by phage P1-mediated three-factor crosses, and the possibility of a *pan* operon was proposed. This suggestion is lent further support by our finding that the *panB* transcript is 1.9 kbp

TABLE 2. Recovery and yield obtained during purification of recombinant KPHMT from *E. coli* Hfr3000 YA139 containing plasmid pCEJ01

Sample	Total protein (mg)	Sp act (mmol min^{-1} mg^{-1})	% Recovery per step	Overall purification (fold)
Crude extract	2,100	0.202		1
DEAE-Sepharose	20	1.133	53	5.6
70% $(NH_4)_2SO_4$	3.5	3.71	57	18.4
Superose 12	1.0	2.72	42	13.5
Heat treatment	0.53	3.89	64	19.3
Mono Q	0.32	3.17	57	15.6

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E. coli KPHMT          GDS-LGMTVQ--GHDS-TLPV
                       **. .*. .  ** .  .*
E. coli SHMT           GDTVLGMNLAHGGHLTHGSPV
S. typhi SHMT          GDTVLGMNLAQGGHLTHGSPV
B. japonicum SHMT      GDTFMGLDLAAGGHLTHGSPV
C. jejuni SHMT         GDKILGMDLSHGGHLTHGAKV
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FIG. 6. Alignment of KPHMT with bacterial serine hydroxymethyltransferases (SHMT). A region of *E. coli* KPHMT (residues 44 to 60) is shown aligned with internal sequences of SHMT from *Campylobacter jejuni* (5), *E. coli* (20), *S. typhimurium* (27), and *Bradyrhizobium japonicum* (23). An asterisk indicates an identical residue, and a period indicates a similar one. Gaps (−) have been introduced into the KPHMT sequence to improve the alignment.

(Fig. 4), significantly larger than the size of the *panB* gene. However, since nothing is known about the physical characteristics of the proteins encoded by *panC* or *panD* (and thus the sizes of the corresponding genes), whether this transcript could encode all three genes or only two requires further investigation. What can be concluded is that the operon is strongly expressed in actively growing cultures of *E. coli*, which would be expected given the requirement for pantothenate-containing cofactors for lipid synthesis.

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Genetic and Biochemical Analyses of Pantothenate Biosynthesis in *Escherichia coli* and *Salmonella typhimurium*

JOHN E. CRONAN, JR.,* KENNETH J. LITTEL,† AND SUZANNE JACKOWSKI‡

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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Pantothenate (*pan*) auxotrophs of *Escherichia coli* K-12 and *Salmonella typhimurium* LT2 were characterized by enzymatic and genetic analyses. The *panB* mutants of both organisms and the *pan-6* ("*panA*") mutant of *S. typhimurium* are deficient in ketopantoate hydroxymethyltransferase, whereas the *panC* mutants lack pantothenate synthetase. *panD* mutants of *E. coli* K-12 were previously shown to be deficient in aspartate 1-decarboxylase. All mutants showed only a single enzyme defect. The finding that the *pan-6* mutant was deficient in ketopantoate hydroxymethyltransferase indicates that the genetic lesion is a *panB* allele. The *pan-6* mutant therefore is deficient in the utilization of α-ketoisovalerate rather than the synthesis of α-ketoisovalerate, as originally proposed. The order of the *pan* genes of *E. coli* K-12 was determined by phage P1-mediated three-factor crosses. The clockwise order was found to be *aceF panB panD panC tonA* on the genetic map of *E. coli* K-12. The three-factor crosses were greatly facilitated by use of a closely linked Tn*10* transposon as the outside marker. We also found that supplementation of *E. coli* K-12 auxotrophs with a high concentration of pantothenate or β-alanine increased the intracellular coenzyme A level two- to threefold above the normal level. Supplementation with pantoate or ketopantoate resulted in smaller increases.

Although study of pantothenate biosynthesis in *Escherichia coli* and *Salmonella typhimurium* began over 20 years ago, our knowledge of the mechanism and regulation of this pathway remains fragmentary. For example, the organization of the genes within the cluster of genes at min 3 to 4 of the genetic maps of both organisms is unknown (3, 24). Also, since the pantothenate auxotrophs studied biochemically were derived from the genetically cryptic W strain of *E. coli* (15, 25, 30), the genetic and enzymological analyses have not been complementary.

In this paper, we report the enzymatic characterization of the pantothenate (*pan*) auxotrophs of *E. coli* K-12 and *S. typhimurium* LT2 and the order of genes within the clustered *pan* loci of *E. coli* K-12. We also demonstrate that the level of coenzyme A (CoA) in *E. coli* can be increased about two- to threefold by excess supplementation with pantothenate or β-alanine, whereas addition of pantoate gives a much smaller increase in CoA levels.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* strains used were derivatives of strain K-12 except for two derivatives of *E. coli* W used in cross-feeding experiments. The *S. typhimurium* strains were all derivatives of strain LT2. Table 1 lists the relevant genotypes and sources of these strains. The minimal medium used was medium E (27). The concentrations of supplements added (when specified) were glucose or sodium succinate, 0.4%; vitamin-free casein hydrolysate, 0.1%; thiamine, 1 mg/liter; L-amino acids, 10 mg/liter; tetracycline-hydrochloride, 10 mg/liter; and adenine, 50 mg/liter. The concentrations of pantothenate and its precursors were varied as described below. Glucose was the major carbon source except when scoring *ace* strains, where succinate was used. All cultures were grown at 37°C with vigorous shaking. Growth was followed turbidimetrically (Klett colorimeter, green filter).

Genetic techniques. The procedures for P1 transduction (7) and construction of a P1 pool of random Tn*10* insertions (6) have been previously described. The *zad*::Tn*10* insertion was obtained by transduction of a *panB* strain, Hfr 3000 YA139, to *pan*$^+$ Tet^r with the P1 pool. This insertion was then used to construct a number of *pan* strains. Some examples are the construction of strains SJ2, CY271, CY278, and CY279. Strain SJ2 was derived from strain Hfr 3000 YA139 by transduction to Tet^r with a phage stock grown on the original insertion strain and screening for a *panB* recombinant. Strain CY271 was the product of a cross of strain AB354 with a P1 stock grown on strain SJ2 and selection on a medium containing tetracycline and pantoate but lacking β-alanine. Strain CY278 was constructed by using the SJ2 phage stock to transduce a prototrophic strain to Tet^r on minimal medium. A P1

† Present address: Department of Microbiology, Iowa State University, Ames, IA 50010.

‡ Present address: Department of Biochemistry, St. Jude Childrens Research Hospital, Memphis, TN 38101.

stock was grown on strain CY279 and used to transduce strain AT1371 to Tet^r on pantothenate-containing medium. The Tet^r recombinants were then screened for the rare (2 of 145) Tet^r strains that remained *panC*. Strain CY278 is one of these Tet^r *panC* recombinants. The other *pan* strains were constructed by analogous crosses (Table 1).

Cross-feeding assays. Cross-feeding between *pan* auxotrophs was assayed on plates of medium E supplemented with glucose (0.4%), thiamine (1 mg/liter), and any necessary amino acids at 10 mg/liter. In some experiments no pantothenate was added, whereas in others 0.05 μM pantothenate was present. The cross-streaks contained about 10^8 washed bacteria per ml.

Enzyme assays. Extracts were made from cells grown to late log phase in medium E supplemented with glucose, thiamine, casein hydrolysate, any required amino acids, and pantothenate (2 μM). The cells were harvested, suspended in 0.1 M potassium phosphate containing 10 mM 2-mercaptoethanol (pH 6.8), disrupted in a French press at 18,000 lb/in^2, and centrifuged (15,000 × *g* for 10 min). The supernatant was dialyzed against the same buffer overnight at 4%.

Aspartate 1-decarboxylase was assayed as previously described (7) except that the entire deproteinized supernatant (0.25 ml) was applied to the preadsorbant area of a Whatman L6 thin-layer plate. A unit of decarboxylase activity is a picomole of β-alanine formed per minute. Pantothenate synthetase was assayed by conversion of β-[1-^{14}C]alanine to pantothenate by the method of Miyatake et al. (18) with the following modifications. The pH was lowered to pH 8.1 (the *S. typhimurium* enzyme was inactive at pH 10), the incubation temperature was 37°C, and the β-alanine concentration was 3 mM (1,100 dpm/nmol). We also decreased the reaction volume to 0.1 ml and added KCl to 15 mM. It should be noted that the *S. typhimurium* enzyme reaction was linear only up to 10 μg of crude extract protein per reaction, whereas the *E. coli* K-12 enzyme reaction was linear to 50 μg per reaction.

Ketopantoate hydroxymethyltransferase was assayed by incorporation of H^{14}CHO (10 mCi/mmol; New England Nuclear Corp.) into ketopantoate. The assay mixture was identical to that of Teller et al. (25), except that the assay volume was reduced to 50 μl and the specific activity of the H^{14}CHO was increased. The dimedon assay of Taylor and Weissbach (24) replaced the cumbersome method of assaying HCHO incorporation previously used (25). After incubation of the assay mixture at 37°C (in a 13 by 100-mm screw-cap tube), 0.5 ml of 0.6 M sodium acetate buffer, pH 4.5 (containing 0.4 M HCHO), and 0.45 ml of 0.4 M dimedon in 50% ethanol were added. The tubes were capped, placed in boiling water for 5 to 10 min, and cooled. $CHCl_3$ (3 ml) was added, the tubes were centrifuged, and 0.2 to 0.4 ml of the upper (water-ethanol) phase (1 ml) was removed for scintillation counting. This is a modification of the Taylor and Weissbach (24) method in that formation of the dimedon adduct was used to remove unreacted H^{14}CHO from the water phase rather than to assay production of H^{14}CHO. The substitution of $CHCl_3$ for toluene in the dimedon extraction facilitated pipetting of the aqueous phase. This procedure removed >94% of the unreacted H^{14}CHO from the aqueous phase. Similar results were found using CCl_4.

All enzyme assays were linear with respect to protein concentration and time. Dialyzed extracts showed essentially absolute dependencies on each of the substrates of the reaction. Scintillation counting was done in PCS (Amersham Corp.) with an efficiency of 88 to 91%. Protein concentrations were determined by a biuret procedure (12), using bovine serum albumin as the standard.

Determination of CoA levels. The strains were grown overnight from a single colony in medium E containing glucose, casein hydrolysate (vitamin-free), the required amino acids, and 1 μM pantothenate. The cultures were harvested on a membrane filter (Millipore HA, 0.45 μm), washed with medium E, and resuspended in the same growth medium lacking pantothenate. Pantothenate or its precursors were added as specified. The cultures (50 ml) were grown from 5 × 10^7 to 10^8 cells/ml until mid to late log phase (5 × 10^8 to 8 × 10^8 cells/ml) with vigorous aeration. The cultures were harvested well before the onset of stationary phase (which can decrease CoA levels [1]). The cultures were harvested by membrane filtration, washed with medium E, eluted from the filter with medium E, and concentrated by centrifugation. The cell pellet was suspended in 0.9 ml of water and placed on ice. Formic acid (0.1 ml of 1 M) was added (final pH 2.3); after 30 min on ice, cellular debris was removed by centrifugation, and the supernatant was neutralized with ammonium hydroxide (14). CoA concentrations were determined by the cycling assay of Allred and Guy (2) after reduction of the samples with dithiothreitol. This assay measures the total cellular CoA, including CoA disulfide, the mixed disulfide of CoA with glutathione, and acetyl-CoA (2, 14).

RESULTS

Preliminary classification of *pan* auxotrophs. The auxotrophs of *E. coli* K-12 and *S. typhimurium* LT2 were of three distinct classes: *panB*, *panC*, and *panD* (Table 1 and Fig. 1). *panB* mutants grow on pantothenic acid, pantoic acid (or its lactone), and ketopantoic acid but do not respond to β-alanine or α-ketoisovaleric acid. *panC* mutants grow only on pantothenic acid; none of the other compounds either alone or in combination (e.g., β-alanine plus pantoic acid) elicits growth. *panD* mutants of *E. coli* K-12 respond only to pantothenate or β-alanine (7). Mutants of *S. typhimurium* responding to β-alanine have been reported (19), but these strains have not been available to other laboratories. The *S. typhimurium* collection contains one strain, *pan-6*, that responds to α-ketoisovalerate (or valine) as well as to each of the compounds that support growth of *panB* strains. This strain was thought to represent a fourth genetic locus (called *panA*), but enzymatic analysis (see below) indicated that the *pan-6* lesion is an allele of the *panB* locus.

Cross-feeding experiments among these strains and the biochemically characterized *E. coli* W strains (15, 25, 30) supported this classification. *panC* strains cross-fed all other auxo-

TABLE 1. Bacterial strains

Strain	Relevant genotype	Source
E. coli K-12		
AB352	*pan*$^+$	E. Adelberg (CGSC[a])
AB354	*panD2*	E. Adelberg (CGSC)
AB2638	*panC5*	E. Adelberg (CGSC)
AT1371	*panC4*	A. L. Taylor (CGSC)
CY257	*panB6*	Laboratory strain (7)
CY271	*panB6* Tn*10*[b]	P1(SJ2) × AB354[c]
CY276	*panD2* Tn*10*	P1(CY279) × AB354
CY277	*panD2 panB6*	P1(CY276) × CY257
CY278	*panC4* Tn*10*	P1(CY279) × AT1371
CY279	*pan*$^+$ Tn*10*	P1(SJ2) × WN1
CY280	*pan*$^+$ Tn*10*	P1(CY279) × AB354
CY282	*pan*$^+$ Tn*10 aceF10*	P1(CY279) × AceF10
CY285	*panD2 panB6* Tn*10*	P1(CY277) × AB354
SJ2	*panB6* Tn*10*	See text
Hfr 3000 YA139	*panB6*	F. Jacob (7)
AceF10	*aceF10*	U. Henning (CGSC)
UB1005	*pan*$^+$	Laboratory strain
WN1	*pan*$^+$	Laboratory strain
E. coli W		
M99-1	*panC*	B. Davis (16)
M99-2	*panD*	B. Davis (16)
S. typhimurium LT2		
LT2	*pan*$^+$	J. Roth
pan-6[d]	*panB6*	K. Sanderson (10)
pan-4	*panB4*	K. Sanderson (10)
pan-2	*panC2*	K. Sanderson (10)

[a] Coli Genetic Stock Center, Yale University, New Haven, Conn.
[b] The Tn*10* in all cases is the *zad*::Tn*10* discussed in the text.
[c] The donor in the P1 transduction is the strain in parentheses.
[d] This strain has formerly been referred to as *panA* (see text).

trophs but were not cross-fed. *panB* and *panD* auxotrophs mutually cross-fed, and all the other auxotrophs cross-fed the *pan-6* strain. No mutants in the same nutritional class were able to cross-feed one another.

Enzymatic analysis of pantothenate auxotrophs. The results of enzymatic analysis (Table 2) were those expected from the cross-feeding and nutritional data and previous enzymatic analysis of the *E. coli* W auxotrophs (15, 25, 30). The *panC* mutants lacked pantothenate synthetase, and *panB* mutants were deficient in ketopantoate hydroxymethyltransferase. As previously demonstrated (7), *panD* mutants lack

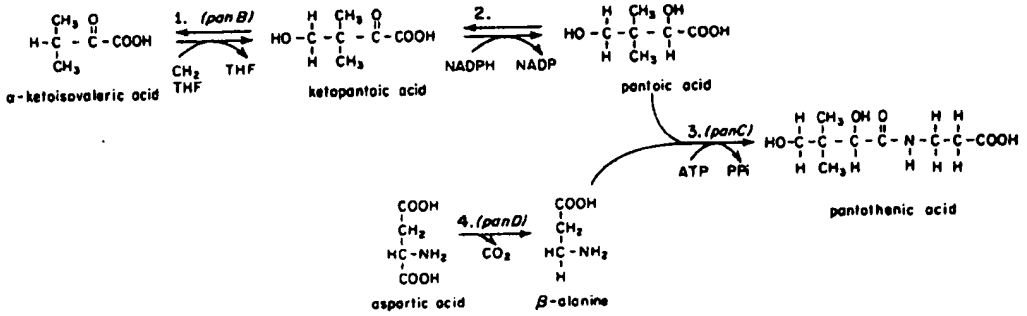

FIG. 1. Pantothenate synthesis in *E. coli* and *S. typhimurium*. The reaction steps are numbered, and the gene loci believed to code for the enzymes catalyzing the steps are given in parentheses. Step 1 is catalyzed by ketopantoate hydroxymethyltransferase; step 2 could occur on free acid (as drawn) by using ketopantoate reductase or as the lactone with ketopantoyl lactone reductase (28, 29). Step 3 is catalyzed by pantothenate synthetase, and step 4 is catalyzed by aspartate 1-decarboxylase. The systematic name for ketopantoic acid is 2-keto-4-hydroxy-3, 3-dimethylbutyric acid. See the text for discussion of this scheme.

TABLE 2. Enzymatic characterization of pantothenate auxotrophs[a]

Strain	*pan* allele	Sp act (U/mg of protein)		
		Transferase	Synthetase	Decarboxylase
E. coli K-12				
AB352	+	2.5	8.1	3.1
CY271	*B*	0.12	8.7	5.5
AB2836	*C*	2.9	0.002	9.5
AT1371	*C*	3.7	<0.001	3.4
AB354	*D*	3.7	8.4	<0.005
S. typhimurium LT2				
LT2	+	2.7	3.1	2.3
pan-6	"*A*"	0.06	3.1	3.3
pan-4	*B*	<0.01	3.4	5.4
pan-2	*C*	2.5	<0.007	5.5

[a] The enzymes assayed were ketopantoate hydroxymethyltransferase, pantothenate synthetase, and aspartate 1-decarboxylase. A unit of the first two enzymes is 1 nmol of product formed per min, whereas a unit of decarboxylase activity is a 1 pmol of β-alanine formed per min. All of the *E. coli* decarboxylase data except that of strain AB2836 are from reference 7.

aspartate 1-decarboxylase. No pleiotrophic effects were found; each class of auxotroph was deficient in only one enzyme. However, one clarifying result was obtained: the *pan-6* mutant of *S. typhimurium* was severely deficient in ketopantoate hydroxymethyltransferase activity. The activity of this enzyme in crude extracts was very low, and this residual activity was unusually labile to storage and purification on DEAE-cellulose. Although these properties precluded a kinetic analysis, the *pan-6* enzyme was inhibited at high α-ketoisovalerate concentrations (10 to 200 mM), whereas the enzyme from strain LT2 showed normal saturation kinetics (*Km* ~1/mM). The defective ketopantoate hydroxymethyltransferase activity of the *pan-6* mutant indicated that the *pan-6* lesion is in the *panB* gene rather than in a locus concerned with α-ketoisovalerate synthesis as proposed by Demerec and co-workers (10).

Order of the *pan* loci of *E. coli* K-12. The *panB*, *-C*, and *-D* loci are closely clustered at min 3.1 of the *E. coli* K-12 genetic map (3, 7). However, the order of genes within the cluster was unknown due to the lack of a closely linked, readily scored outside marker. We used a Tn*10* transposon inserted very close to (but outside) the *pan* cluster to order these genes. The particular Tn*10* insertion was isolated by transduction of a *panB* strain to *pan*$^+$ Tet^r with a phage P1 stock grown on a pool of insertions formed by transposition from a phage λ Tn*10* vector (6). The insertion chosen was 99.6% linked to the *pan* locus (631 colonies scored) and 32.3% linked to the *aceF* locus (353 colonies scored) by P1 transduction (selection for Tet^r).

We first ordered the Tn*10* insertion in relation to *pan* and the outside markers *aceF* (min 2.7) and *tonA* (min 3.4). If the Tn*10* insertion was in the *aceF* donor strain, prototrophic Tet^r recombinants were readily formed (Table 3, cross 1). However, if the donor was a Tn*10 panB* strain (cross 2), no prototrophic Tet^r recombinants were found. These results were interpreted in the standard manner; formation of Tet^r recombinants always required only two crossover events, whereas formation of Tet^r prototrophs required either two or four crossover events, depending upon the gene order. In cross 1, Tet^r prototrophs were formed by two crossovers, whereas four crossovers were needed to form such recombinants in cross 2. Thus, the data were consistent only with the clockwise order *aceF panB* Tn*10*. A cross similar to cross 1 with a *panC* recipient gave the order *aceF panC* Tn*10* (data not shown). Three-factor crosses using the Tn*10* insertion *panB* and *tonA* were consistent with crosses 1 and 2 and indicated the clockwise order *panB* Tn*10 tonA* (data not shown). The positive selection for the Tn*10* element greatly simplified the scoring of three-factor crosses and avoided the difficulty in scoring *pan* auxotrophs caused by heavy cross-feeding by neighboring *pan*$^+$ colonies.

Crosses were then performed among the three types of *pan* auxotrophs, the donor strain carrying the Tn*10* insertion. The relative differences between the two-crossover and four-crossover classes were 20-fold or greater. Crosses 3 and 4 indicated the order *panB panD* Tn*10*, whereas crosses 5 and 6 gave the order *panD panC* Tn*10*. The relative order of *panB* and *panC* (crosses 7 and 8) was shown to be *panB panC* Tn*10*. Thus, the overall clockwise order was unambiguously *panB panD panC* Tn*10*. This order was confirmed by cross 9, in which the Tn*10* and the *panC*$^+$ allele of the donor were recombined with the *panB*$^+$ and *panD*$^+$ alleles of the recipient with the frequency expected for a two-factor cross. A further confirmation was that strains

TABLE 3. Order of *pan* genes of *E. coli* K-12[a]

Cross	Donor	Recipient	Total Tet^r recombinants formed	Tet^r prototrophic recombinants formed	% Tet^r prototrophs
1	CY282 Tn*10 aceF*	CY257 *panB*	397	447	112.6
2	SJ2 Tn*10 panB*	AceF10 *aceF*	160	0	<0.6
3	CY276 Tn*10 panD*	CY257 *panB*	343	0	<0.3
4	SJ2 Tn*10 panB*	AB354 *panD*	162	24	14.8
5	CY276 Tn*10 panD*	AT1371 *panC*	76	16	21.1
6	CY278 Tn*10 panC*	AB354 *panD*	86	0	<1.2
7	SJ2 Tn*10 panB*	AT1371 *panC*	26	8	26.5
8	CY278 Tn*10 panC*	CY257 *panB*	135	0	<0.7
9	CY277 Tn*10 panD panB*	AT1371 *panC*	480	30	6.3

[a] After adsorption of the transducing phage, half of the washed cells were plated on medium containing both tetracycline and pantothenate (total Tet^r recombinants formed) or tetracycline without pantothenate (Tet^r prototrophic recombinants formed). In crosses 1 and 2, the supplemented plates also contained acetate.

requiring both β-alanine and pantoic acid (*panB panD*) were readily (12% frequency) recovered from the pantothenate-containing plates of cross 3, whereas the colonies from the pantothenate-containing plates of cross 4 were either *panB* (94%) or *panD* (6%); no *panB panD* colonies were found.

Does pantothenate biosynthesis regulate CoA levels? Brown (5) reported that the intracellular CoA levels of *E. coli* and other bacteria could be increased 9.2-fold by supplementation of the culture medium with pantothenate. Alberts and Vagelos (1) showed that the intracellular concentration of CoA could be altered (12- to 15-fold) by the level of pantothenate supplementation in an *E. coli* K-12 *panB* strain. Growth was normal over this range of pantothenate concentrations (1). However, the value (0.1 nmol/mg, dry weight) reported by Brown (5) for the CoA content of *E. coli* grown without pantothenate was much lower than more recent estimates (13, 14); thus, it was unclear if pantothenate biosynthesis regulates the CoA level of this organism. For these reasons we reexamined the effect of various levels of pantothenate and its precursors to determine if the CoA level of *E. coli* could be expanded by excess supplementation with these precursors and if all precursors were equally active. For the purposes of the latter consideration, various *pan* auxotrophs constructed during the mapping experiments were used to ensure entry of the precursors. Wild-type strains of *E. coli* excrete copious amounts of pantothenate (9, 11, 16), causing uptake of added pantothenate and its precursors to be very inefficient.

Supplementation of cultures of *E. coli* pantothenate auxotrophs with 100 μM pantothenate increased the intracellular level of CoA by 2- to 2.5-fold over that found in closely related wild-type strains (Table 4). Supplementation with β-alanine resulted in similar increases in CoA levels. However, supplementation with pantoate or ketopantoate gave only about a 50% increase in CoA levels even in the presence of 100 μM pantoate or ketopantoate. This insensitivity to pantoate supplementation was not due to limitation of the rate of pantoate accumulation, because supplementation of a *panB panD* auxotroph (strain CY285) with high concentrations (100 μM) of both pantoic acid and β-alanine gave the same increase in CoA level as did supplementation with 100 μM pantothenate (data not shown).

DISCUSSION

Enzymatic analysis of the *E. coli* K-12 and *S. typhimurium pan* auxotrophs gave results consistent with those expected from the supplementation and cross-feeding experiments as well as previous work on *E. coli* W (15, 25, 30). The most important result was that the *pan-6* mutant of *S. typhimurium* had a defective ketopantoate hydroxymethyltransferase. Demerec and coworkers (10) concluded that the growth of the *pan-6* strain on α-ketoisovaleric acid or pantothenate indicated a deficiency in the synthesis of α-ketoisovaleric acid. Recently, Cronan (7) pointed out that such a strain would require valine when grown on pantothenate (only 0.1 μM pantothenate is required for growth, thus precluding the possibility of conversion of pantothenate to valine or a valine precursor). Cronan (7) therefore proposed that the *pan-6* ("*panA*") strain contained a mutant ketopantoate hydroxymethyltransferase having an elevated K_m for α-ketoisovaleric acid. Another possibility was that the α-ketovalerate pool utilized for pantothenate was different from that transaminated to valine. However, W. A. Whalen and C. M. Berg (Genetics 97:S112, 1981) recently reported that an *E. coli* strain lacking transaminase C and the enzymes of α-ketoisovalerate synthesis required pantothenate in addition to valine, isoleucine, and leucine. Hence, it is clear that the valine synthetic pathway pro-

TABLE 4. CoA levels in cultures supplemented with pantothenate or its precursors

Strain	Genotype	Supplement	CoA level (nmol/mg, dry weight)
CY280	*pan*$^+$	None	0.29
AB352	*pan*$^+$	None	0.33
AB354	*panD*	1 μM pantothenate	0.34
AB354	*panD*	100 μM pantothenate	0.74
AB354	*panD*	10 μM β-alanine[a]	0.48
AB354	*panD*	100 μM β-alanine	0.85
CY271	*panB*	1 μM pantothenate	0.36
CY271	*panB*	100 μM pantothenate	0.60
CY271	*panB*	1 μM pantoate	0.48
CY271	*panB*	100 μM pantoate	0.49
CY271	*panB*	1 μM ketopantoate	0.48
CY271	*panB*	100 μM ketopantoate	0.49

[a] The unusually high requirement for β-alanine of strain AB354 is due to at least two factors. First, the threonine and leucine required by this strain inhibited the rate of uptake about twofold (presumably through competition for the neutral amino acid transport system). Second, *thr*$^+$ *leu*$^+$ derivatives of AB354 required about twice the level of β-alanine for full growth as that required by strain SJ16 (11) and other strains carrying the *panD2* lesion, although the initial rate and affinity of β-alanine transport were normal. Thus, strain AB354 seems deficient in the utilization of β-alanine (or its derivatives).

vides the α-ketoisovalerate for pantothenate synthesis.

The *pan-6* mutant is severely deficient in ketopantoate hydroxymethyltransferase activity, although the activity is significantly greater than that of the *panB* strain tested (Table 2). Unfortunately, we were unable to measure the K_m of the *pan-6* transferase due to its low activity, instability, and inhibition by high α-ketoisovalerate concentrations. However, this abnormal inhibition did indicate an altered binding of α-ketoisovalerate by the *pan-6* enzyme. These data demonstrate that the *pan-6* mutant is an allele of the *panB* locus, and the "*panA*" locus should be deleted from the genetic map of *S. typhimurium* LT2. It should be noted that Demerec et al. (10) reported that the *pan-6* and *panB* mutants were genetically complementary by abortive transduction. However, no data were given, and complementation can readily be attributed to intragenic rather than intergenic complementation. The ketopantoate hydroxymethyltransferase of *E. coli* K-12 is a homodecameric enzyme (25), and therefore complementation due to subunit mixing seems likely.

E. coli K-12 *pan* auxotrophs fall clearly into three classes, *panB*, *panC*, and *panD*, by physiological, enzymatic, and genetic criteria. The three genes are closely linked in the clockwise order *aceF panB panD panC tonA*. These three genes, perhaps together with genes coding for enzymes involved in converting pantothenate to 4'-phosphopanthetheine, may comprise an operon. However, no evidence currently exists for or against this possibility.

We have found that supplementation of *E. coli* K-12 *pan* auxotrophs with pantothenate or β-alanine increases the CoA pool two- to threefold over the normal level. The 10-fold variations in CoA levels that can be engendered in *E. coli pan* auxotrophs (1, 11, 20, 26), therefore, are the product of both low CoA levels due to pantothenate limitation and elevated CoA levels due to pantothenate supplementation.

The rate-limiting step in the conversion of pantothenate to CoA appears to be the phosphorylation of pantothenate by pantothenate kinase (11). This conclusion is based on the copious excretion of pantothenate by *E. coli* (9, 11, 16). Over 90% of the pantothenate synthesized in *E. coli* is excreted rather than utilized for CoA and acyl carrier protein synthesis (11). For this reason the reactions of pantothenate biosynthesis cannot be considered in the usual sense to limit the rate of CoA synthesis (11). However, supplementation of the growth medium with excess pantothenate did increase the CoA level, implying that regulation at the pantothenate kinase step is not absolute. The external supplement presumably increases the intracellular pantothenate level (~10 μM calculated from reference 11) by facilitated diffusion (17), which in turn increases the rate of pantothenate phosphorylation.

β-Alanine supplementation increased CoA levels markedly, whereas supplementation with excess pantoate or ketopantoate was much less effective (Table 4). This finding argues that cellular synthesis of β-alanine limits pantothenate production in wild-type strains but that the synthesis of pantoate is sufficient. Indeed, bioassay of the medium of the wild-type strain UB1005 suggested that excess pantoate is produced during growth. The medium supported full growth of a *panB* strain at a fivefold-greater dilution than that required for full growth of

either a *panC* or a *panD* strain. This result implies accumulation of free pantoate (or ketopantoate or both) in the medium.

Loewen (14) measured the CoA levels of *E. coli* B grown without pantothenate. We obtained two- to threefold greater levels of CoA in our *E. coli* K-12 strains upon supplementation with excess pantothenate or β-alanine. These levels of CoA were very similar to those found in other strains of *E. coli* supplemented with excess pantothenate or β-alanine (1, 5, 11, 20, 26). The unusually low level of CoA reported for *E. coli* grown in the absence of pantothenate by Brown (5) seems spurious and can probably be attributed to the condition of growth (1). It should be noted that Loewen (13) recently reported extremely high levels of CoA in several *E. coli* K-12 strains. However, the original data in that paper are inconsistent with the tabulated values and seem in agreement with the values reported in his previous paper (14). Our data and those of others (1, 11, 20) demonstrate that *E. coli* grows normally over a 10-fold range of CoA levels and that strict regulation of the CoA pool is not required for growth.

The apparent lack of regulatory mechanisms controlling the rate of pantothenate synthesis in *E. coli* could be expected because strict regulation of the intracellular level of CoA is not required for growth. Moreover, the pantothenate synthetic pathway requires only a few enzymes, and the mass of cellular material consumed in synthesis of pantothenate is low compared with the mass of the amino acids from which pantothenate is derived. The synthesis of pantoate consumes 10% of the amount of α-ketoisovalerate used in valine synthesis, and the synthesis of β-alanine consumes <2% of the cellular aspartate. For these reasons, the metabolic cost of regulating this pathway could exceed the cost of the seemingly wasteful overproduction and excretion of pantothenate.

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D-Pantothenate Synthesis in *Corynebacterium glutamicum* and Use of *panBC* and Genes Encoding L-Valine Synthesis for D-Pantothenate Overproduction

HERMANN SAHM AND LOTHAR EGGELING*

Institut für Biotechnologie, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

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D-Pantothenate is synthesized via four enzymes from ketoisovalerate, which is an intermediate of branched-chain amino acid synthesis. We quantified three of these enzyme activities in *Corynebacterium glutamicum* and determined specific activities ranging from 0.00014 to 0.001 μmol/min mg (protein)$^{-1}$. The genes encoding the ketopantoatehydroxymethyl transferase and the pantothenate synthetase were cloned, sequenced, and functionally characterized. These studies suggest that *panBC* constitutes an operon. By using *panC*, an assay system was developed to quantify D-pantothenate. The wild type of *C. glutamicum* was found to accumulate 9 μg of this vitamin per liter. A strain was constructed (i) to abolish L-isoleucine synthesis, (ii) to result in increased ketoisovalerate formation, and (iii) to enable its further conversion to D-pantothenate. The best resulting strain has *ilvA* deleted from its chromosome and has two plasmids to overexpress genes of ketoisovalerate (*ilvBNCD*) and D-pantothenate (*panBC*) synthesis. With this strain a D-pantothenate accumulation of up to 1 g/liter is achieved, which is a 10^5-fold increase in concentration compared to that of the original wild-type strain. From the series of strains analyzed it follows that an increased ketoisovalerate availability is mandatory to direct the metabolite flux into the D-pantothenate-specific part of the pathway and that the availability of β-alanine is essential for D-pantothenate formation.

D-Pantothenate is a water-soluble vitamin required as a pharmaceutical and a feed additive. About 4,000 tons of pantothenate are produced annually (48). The present method of production depends for the most part on chemical synthesis from bulk chemicals. However, this synthesis requires the optical resolution of racemic intermediates. Therefore, a variety of routes have been assayed to improve its synthesis, including enzyme conversions (41). One of the processes of D-pantothenate synthesis uses a lactonohydrolase activity of *Fusarium oxysporum*, which catalyzes the stereospecific hydrolysis of chemically made D,L-pantolactone to generate D-pantolactone as a chiral building block for its further chemical conversion to D-pantothenate (19). Therefore, there is still potential for further improving D-pantothenate production, for instance, by its direct microbial synthesis.

In *Escherichia coli* the specific biosynthesis pathway of this vitamin consists of only four steps (Fig. 1). The first reaction, catalyzed by the ketopantoatehydroxymethyl transferase, uses the L-valine intermediate 2-ketoisovalerate to generate ketopantoate, which is reduced to D-pantoic acid. An aspartate-α-decarboxylase activity generates β-alanine, which is ligated with pantoic acid to yield D-pantothenate. The respective enzymes of *E. coli* and *Salmonella typhimurium* have been characterized, and the corresponding genes have been identified (11, 15). Also for *Bacillus subtilis*, transferase and ketopantoate reductase activities have been demonstrated (1). In general, three different mechanisms of β-alanine formation are thought to be present in microorganisms (41).

We are interested in metabolite flux analysis in the gram-positive bacterium *Corynebacterium glutamicum* (25). This bacterium is used for the large-scale production of L-lysine and L-glutamate (22). It has a high capacity to supply precursor metabolites (26), and its molecular physiology of amino acid synthesis has been analyzed in detail (36). We have also developed strains producing L-isoleucine (7), the synthesis of which uses enzymes in part identical to those required for the synthesis of L-valine (Fig. 1). Due to the linkage of the branched-chain amino acid synthesis with the short reaction sequence of D-pantothenate synthesis, the analysis of D-pantothenate formation with *C. glutamicum* is an attractive target. Moreover, a closely related bacterium, *Brevibacterium ammoniagenes*, has already been reported to accumulate coenzyme A, which is synthesized from D-pantothenate (42). In the present work, we analyze enzymes and genes involved in D-pantothenate synthesis by *C. glutamicum* and study their use, together with genes of branched-chain amino acid synthesis, for the direct microbial synthesis of D-pantothenate.

MATERIALS AND METHODS

Strains, plasmids, and cultivations. The strains and plasmids used are shown in Table 1. *C. glutamicum* was grown on brain heart infusion medium or minimal medium CGXII (20). *E. coli* was grown in Luria broth or minimal medium M9 (45). The cultivations of strains containing the pEKx2 plasmids were done in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) added 5 h after inoculation.

Metabolite quantifications. D-Pantothenate was quantified in a microbiological assay with *C. glutamicum* R127::*panC* (this work). For this purpose, cells of this strain were precultivated overnight on brain heart infusion medium (with 25 μg of kanamycin [Difco]), washed twice with 9 g of NaCl per liter, and inoculated into minimal medium CGXII (with 25 μg of kanamycin) to obtain an initial optical density at 600 nm (OD_{600}) of 0.5. This served to deprive the cells of D-pantothenate. Although D-pantothenate had not been supplied, growth of the cells was possible up to an OD_{600} of about 20 (the control strain, *C. glutamicum* R127, reaches an OD_{600} of about 40). One milliliter of the pantothenate-deprived culture (taken after 30 h) was mixed with 700 μl of glycerol and stored at −70°C. Sixty-microliter aliquots of these stocks were used to inoculate assay tubes. These assay tubes (Falcon 2057; Becton and Dickinson) contained 3 ml of four-thirds concentrated CGXII medium (with 25 μg of kanamycin), 1 ml of sterile filtered D-pantothenate sample, and *C. glutamicum* R127::*panC* (60 μl). Tubes were cultivated for 40 h at 30°C with shaking, and the OD_{600} was determined. On the basis of results from this procedure, growth is linearly dependent

* Corresponding author. Mailing address: Institut für Biotechnologie, Forschungszentrum Jülich GmbH, 52428 Jülich, Germany. Phone: 49 2461 61 5132. Fax: 49 2461 61 2710. E-mail: l.eggeling@fz-juelich.de.

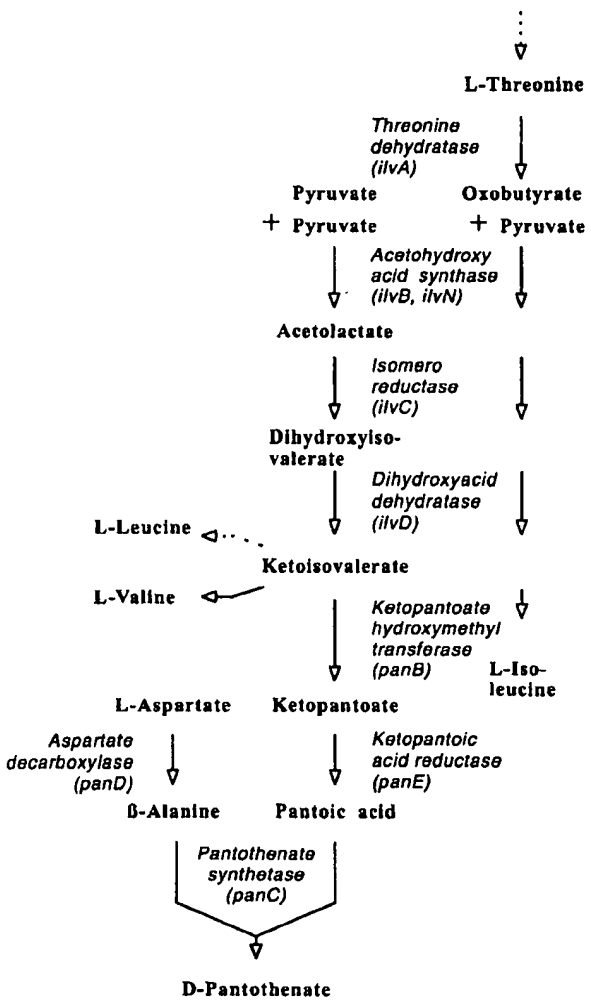

FIG. 1. The pathway of D-pantothenate biosynthesis and its integration into the synthesis of branched-chain amino acids.

on the concentration of D-pantothenate over a broad concentration range (Fig. 2), which is a clear advantage compared to the standard D-pantothenate determination with *Lactobacillus plantarum* ATCC 8014 according to the U.S. Pharmacopoeial Convention. When assays for one sample were repeated, the standard deviation for 10 ng of D-pantothenate per ml was ± 0.9 ng. The assay is linear in the range from 0 to 100 ng of D-pantothenate per assay (4 ml). In the case of nonlinear growth obtained with new glycerol stock cultures of the *panC* mutant, the inoculum varied between 60 and 100 μl.

L-Valine and β-alanine were quantified by automated precolumn derivatization with *ortho*-phthaldialdehyde (24), followed by reversed-phase chromatography with fluorometric detection (model HP LC1090; Hewlett Packard). α-Ketoisovalerate was derivatized with diaminomethoxybenzole (12) and again quantified by reversed-phase chromatography and fluorometric detection.

Enzyme activity determinations. A crude extract of cells taken from the late-exponential phase was prepared by sonication. The level of ketopantoate-hydroxymethyl transferase activity was determined by quantifying the ketoisovalerate formation from ketopantoate. The assay mixture consisted of 71 mM potassium phosphate (pH 6.8), 1 mM $MgSO_4$, 3.6 mM ketopantoate, and 0.71 mM tetrahydrofolate. The reaction was started by the addition of the crude extract, which was equilibrated prior to use on PD10 columns (LKB-Pharmacia) with 100 mM potassium phosphate (pH 6.8), and was run at 37°C for 60 min.

The pantothenate synthetase activity was assayed, with minor modifications, as described previously (29). The assay mixture consisted of 100 mM Tris-HCl (pH 10), 10 mM $MgSO_4$, 5 mM D,L-pantoate, 5 mM β-alanine, and 10 mM ATP. After the addition of the crude extract the assay mixture was incubated at 30°C for 40 min, and the assay was then terminated by the addition of 5 volumes of isatoic acid anhydride (to a concentration of 3.2 mM in dimethylformamide). This enabled the quantification of pantothenate by reversed-phase chromatography as described by Julliard (18).

The aspartate α-decarboxylase activity was quantified by β-alanine formation in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5), 5 mM EDTA (pH 7.5), and 5 mM L-aspartate. The reaction was started by the addition of the crude extract to the mixture and was run for 60 min at 37°C.

Gene bank and sequence analysis. The gene bank used was as described previously (32). The sequence for both strands of the 2,164-bp fragment was determined by the dideoxy chain termination method on subclones derived from exonuclease treatment of pUR1.1 and pUR1.2. Additional sequence information covering *xylB* was obtained by primer walking.

Plasmid constructions. All plasmid constructions were done in *E. coli* DH5αmcr. Plasmid pJC1*ilvBNCD* was obtained by ligating a 2.6-kb *Xba*I fragment containing *ilvD* into the *Bam*HI site of pKK5 (2). To obtain pECM3*ilvBNC* a 5.7-kb fragment of pKK5 encompassing *ilvBNC* was cloned into the *Eco*RV site of pECM3. Additionally, a 5.7-kb *Xba*I fragment (*ilvBNC*) of pKK5 and a 3.1-kb *Xba*I fragment (*ilvD*) were ligated with *Eco*RV-digested pECM3 to yield pECM3 *ilvBNCD*. Plasmid pEC7*panD* was constructed by ligating a 900-bp *Pvu*II fragment of pDKS1, containing *panD* of *E. coli* (35), with *Sma*I-digested pEC7. To construct pEKEx2*panBC*, the 5′ region of *panB* was amplified with the primers 5′GATCGTCGACCATCACATCTATACTCATGCCC and 5′ACCCGATGTG GCCGACAACC. The resulting PCR fragment was treated with *Sal*I and *Eco*RI and ligated with the identically treated pEKEx2. The plasmid obtained was cleaved with *Eco*RI and ligated with the 1.8-kb *Eco*RI fragment of pUR1, containing the 3′ end of *panB* and *panC*.

Strain constructions. To construct the *ilvA* deletion mutant of *C. glutamicum* ATCC 13032, the 242-bp *Bgl*II fragment of *ilvA* was deleted in pBM21 (30). Subsequently, the fragment with the deletion was excised as a 1.3-kb *Eco*RI

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. coli		
S17-1	Mobilizing strain	43
SJ2	*panB* mutant	5
DV39	*panC* mutant	47
C. glutamicum		
ATCC 13032	Wild type	
ATCC 13032 Δ*ilvA*	*ilvA* deletion mutant	This work
R127	Restriction negative	23
R127::orf1	ORF 1 integration mutant	This work
R127::*panC*	*panC* integration mutant	This work
L. plantarum		
ATCC 8014	Pantothenate auxotroph	
Plasmids		
pJC1	*E. coli-C. glutamicum* shuttle vector, Kan^r	3
pZ1	*E. coli-C. glutamicum* shuttle vector, Kan^r	27
pUR1	pBR322 with 9.3-kb chromosomal *Sau*IIIA fragment encompassing *panBC* and *xylB*	This work
pUR1.1	pUC19 with 2.4-kb *Ssp*I/*Pvu*II fragment encompassing *panB*	This work
pUR1.2	pUC18 with 3.9-kb *Ssp*I/*Sal*I fragment encompassing *panBC*	This work
pEC7*panD*	pEC7 with 900-bp fragment encompassing *panD* of *E. coli*	This work
pEC7	*E. coli-C. glutamicum* shuttle vector, Cm^r	10
pJC1*ilvBNCD*	pKK5 with 2.6-kb *Xba*I fragment encompassing *ilvD*	This work
pJC1*ilvBNC*	pJC1 with 5.7-kb *Hin*dIII/*Eco*RI fragment encompassing *ilvBNC*	This work
pKK5	pJC4 with 5.7-kb *Hin*dIII/*Eco*RI fragment encompassing *ilvBNC*	2
pECM3*ilvBNCD*	pECM3 with 5.7-kb *Xba*I fragment encompassing *ilvBNC* and 3.1-kb *Xba*I fragment encompassing *ilvD*	This work
pECM3*ilvBNC*	pECM3 with 5.7-*Hin*dIII/*Eco*RI fragment encompassing *ilvBNC*	This work
pECM3	Shuttle vector, derived from pECM2, Cm^r	16
pZ1*panBC*	pZ1 with 3.5-kb *Sca*I/*Sal*I fragment encompassing *panBC*	This work
pZ1*panC*	pZ1 with 2.4-kb *Bst*EII/*Sal*I fragment encompassing *panC*	This work
pEKEx2	Expression vector, Kan^r *Ptac*	9

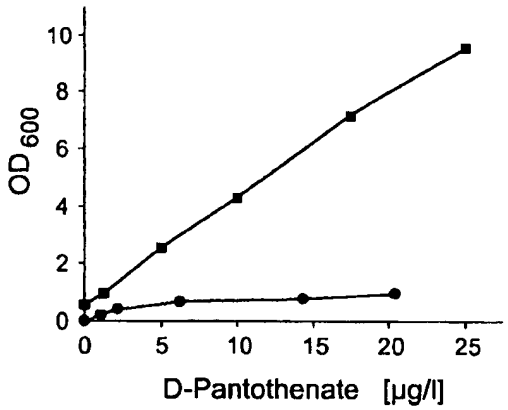

FIG. 2. D-Pantothenate quantification for *C. glutamicum*::*panC* (■) and *L. plantarum* (●). The D-pantothenate concentration given is the final concentration in the assay.

fragment, which was ligated with pK19mobsacB (39). The resulting mobilizable *E. coli* vector enabled the transfer of the deletion into the chromosome of *C. glutamicum* by two rounds of positive selection. The deletion was confirmed by PCR.

To construct the *panC* insertion mutant of *C. glutamicum* R127, an internal 168-bp *panC* fragment was amplified with the primers 5'GTTCGCACCCGATG TGGAGG and 5'ATGCACGATCAGGGCGCACC. The fragment was cloned into the *Sma*I site of pUC18 with the SureClone ligation kit (Amersham), subsequently excised as an *Eco*RI/*Sal*I fragment, and finally ligated with *Eco*RI/*Sal*I-treated pK18mob (39). The resulting vector was transferred to *C. glutamicum* via conjugation (38), and kanamycin-resistant transconjugants were obtained. One strain selected was termed *C. glutamicum* R127::*panC*. Its D-pantothenate auxotrophy was verified, as well as the vector integration into the chromosome.

To construct the open reading frame (ORF) 1 insertion mutant of *C. glutamicum* R127, an internal 202-bp ORF 1 fragment was amplified with the primers 5'GATCGAATTCCCGATTAAATCGCGGAGACGG and 5'GATCGTCGAC CTTTGCTGCCGATTCAAGTG. The fragment was digested with *Eco*RI and *Sal*I and ligated with the *Eco*RI/*Sal*I-treated pK19mobsacB. This vector was used to construct *C. glutamicum* R127::orf1, whose correct integration of the vector into ORF 1 was verified via PCR.

Nucleotide sequence accession number. The sequence for both strands of the 2,164-bp fragment was deposited in the EMBL and GenBank databases under accession no. X96580.

RESULTS

Cloning and sequence analysis of *panBC*. The *E. coli panB* mutant SJ2 (4) was transformed to ampicillin resistance with genomic DNA of *C. glutamicum* ATCC 13032 ligated with pBR322. This yielded eight plasmids able to restore the growth of SJ2 on minimal medium plates. They were found to contain three different inserts of 9.3, 2.1, and 1.8 kb, respectively. The insert in the largest plasmid, named pUR1 (Fig. 3), was assayed by a Southern blot analysis of *Sca*I-digested chromosomal DNA (data not shown) with the 1.5-kb *Pvu*II/*Sal*I fragment as a probe. This confirmed the origin and structural identity of the large fragment cloned. The isolated plasmids were used to assay for an additional complementation of the *panC* mutation of *E. coli* DV39 (47). Plasmid pUR1 complemented this mutation, whereas the two smaller plasmids failed to do so. To further confine the complementing functions, several subclones were made. Whereas pUR1.1 only complemented the mutation in *E. coli* SJ2, pUR1.2 complemented the mutations of both *E. coli* strains. A nucleotide sequence of 2.2 kb from the insert of pUR1 was determined on both strands, whereas the sequence for an adjacent 1.5-kb part was established on one strand only (Fig. 3).

The sequence analyses identified four ORFs. ORF 1 exhibits no identities with known sequences. Inactivation of ORF 1 in *C. glutamicum* R127::orf1 resulted in a decreased growth rate ($\mu = 0.31\ h^{-1}$; $\mu = 0.38\ h^{-1}$ [for *C. glutamicum* R127]), which could not be restored by the addition of D-pantothenate (data not shown). Interestingly, amino acid residues 64 to 89 encoded by ORF 1 fit exactly to the consensus sequence of the helix-turn-helix motif of LysR-type regulators (40). Therefore, it is proposed that ORF 1 encodes a transcriptional regulator which is functionally not related to D-pantothenate synthesis in *C. glutamicum*. The deduced amino acid sequence encoded by the second ORF (nucleotides 351 to 1166) exhibits a high identity with PanB, as does that encoded by the third ORF (nucleotides 1166 to 2005) with PanC polypeptides. The highest identities are shared with PanB of *Mycobacterium tuberculosis* (52%) and PanC of *Schizosaccharomyces pombe* (45%), respectively. The fourth ORF is located on the strand opposite to that of the *pan* genes. Its deduced polypeptide shows significant homology to xylulokinases (encoded by *xylB*).

Enzyme activity determinations. To functionally characterize the genes, enzyme activity determinations in the homologous background were performed. For this purpose, the *Sca*I/*Sal*I fragment of pUR1.2 was ligated with the *E. coli-C. glutamicum* shuttle vector pZ1 (27) to yield pZ1*panBC* and with the *Bst*EII/*Sal*I fragment to yield pZ1*panC* (Fig. 3). With these plasmids the wild type of *C. glutamicum* was transformed. The resulting recombinant strains were grown on minimal medium, and cells were harvested for activity determinations.

The ketopantoatehydroxymethyl transferase activity (*panB*) was determined in a novel assay based on the quantification of ketoisovalerate formed from ketopantoate (see Materials and Methods). With *C. glutamicum*/pZ1*panBC* a specific activity of

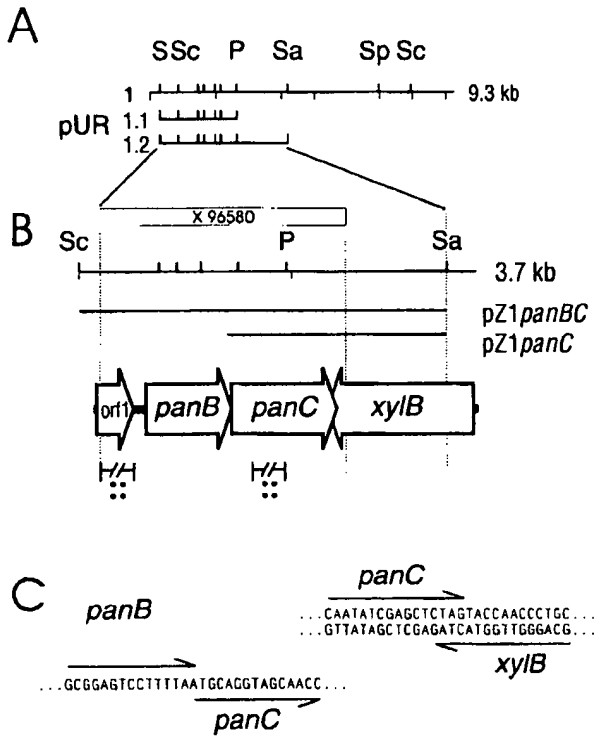

FIG. 3. Overview of the cloned and subcloned chromosomal fragments (A), the sequenced part and organization of the genes (B), and the overlaps of *panB* with *panC* and those of *panC* with *xylB* (C). S, *Sal*I; Sc, *Sca*I; Sa, *Sal*I; P, *Pst*I; Sp, *Sph*I.

TABLE 2. Enzyme activities of D-pantothenate synthesis in *C. glutamicum*, and *E. coli*[a]

Enzyme and strain	Sp act (nmol/min/mg of protein)
Ketopantoate hydroxymethyltransferase	
C. glutamicum	0.14
C. glutamicum pZ1*panBC*	1.9
C. glutamicum pEKEx2*panBC*	1.9
E. coli	3–7
Pantothenate synthetase	
C. glutamicum	1
C. glutamicum pZ1*panBC*	12
C. glutamicum pZ1*panC*	1
E. coli	4.2
Aspartate decarboxylase	
C. glutamicum	0.11
E. coli	0.004–0.17

[a] The *E. coli* data are given for comparison. They are taken from the following references: 4 and 49 (decarboxylase), 29 (synthetase), and 17 and 46 (transferase).

1.9 nmol/min/mg of protein was obtained, whereas the control yielded an activity of 0.14 nmol/min/mg of protein (Table 2). This ~13-fold increase in synthesis confirms the identity of *panB*. It is reported that D-pantothenate and D-pantoate inhibit the transferase activity in *E. coli* (33) and salicylate the enzyme in *S. typhimurium* (34). Therefore, these compounds were included individually at 10 mM concentrations in the enzyme assay with the extract of *C. glutamicum*. Only a marginal effect with D-pantothenate was detected, but D,L-pantoate reduced the transferase activity to 20% and salicylate to 22%.

The activity of the pantothenate synthetase (encoded by *panC*) was determined in *C. glutamicum*/pZ1*panBC*. It is 12 nmol/min/mg of protein, opposed to 1 nmol/min/mg of protein in the control (Table 2). However, with *C. glutamicum*/pZ1*panC*, no increased specific activity was detected. This suggests the organization of *panBC* as an operon, as indicated from the sequence of the cluster.

In addition to the quantification of the transferase and synthase activities, we also assayed *C. glutamicum* for aspartate decarboxylase activity with a novel assay by quantification of β-alanine via high-pressure liquid chromatography. As shown in Table 2, this enzyme has a specific activity of 0.11 nmol/min/mg of protein. For comparison Table 2 also includes decarboxylase, transferase, and synthetase activities for *E. coli*. It can be seen that the enzyme activities in *E. coli* are in the same order of magnitude, except that of the transferase, which is at least 1 order of magnitude higher.

D-Pantothenate formation by the wild type. To assay for D-pantothenate accumulation by *C. glutamicum*, the wild type was grown in minimal medium. In samples of sterile filtered culture supernatants, D-pantothenate was quantified in the assay developed (see Materials and Methods). As can be seen in Fig. 4, there is only a very weak accumulation of maximal 42 nM D-pantothenate in culture supernatants, which is in accord with the low activities and/or a tight control of D-pantothenate synthesis. The unexpected time course of the D-pantothenate concentrations shown in Fig. 4 was verified in a separate experiment. With the wild type of *E. coli* a D-pantothenate accumulation of 3 mg/liter has been reported (14). In an additional experiment a recombinant *C. glutamicum* strain was made and assayed for D-pantothenate formation. This strain was *C. glutamicum*/pZ1*panBC*, which additionally contained the plasmid-encoded L-aspartate decarboxylase (*panD*) of *E. coli* (see Materials and Methods). This strain overexpressing three of the D-pantothenate biosynthesis genes again exhibited a time course of D-pantothenate accumulation almost identical to that of the wild type and also not exceeding 42 nM as the highest concentration. Further engineering was therefore required.

Increased D-pantothenate formation by *ilvA* deletion. We first assayed the consequences of the deletion of the threonine dehydratase gene *ilvA* on a flux increase towards D-pantothenate. This was based on the idea that due to the prevention of L-isoleucine synthesis an increased pyruvate availability could result in increased ketoisovalerate accumulation with further conversion to D-pantothenate (Fig. 1). By the application of two rounds of positive selection for the presence and absence of vector sequences, respectively (39), a *C. glutamicum* wild-type derivative was constructed with the internal 242-bp *Bgl*II fragment of *ilvA* deleted from the chromosome. The D-pantothenate concentration after 24 h of cultivation in minimal medium by the *C. glutamicum ilvA* deletion mutant obtained was 236 nM, which is about a fivefold increase compared to that of the wild type (see above).

L-Valine accumulation by overexpressing *ilv* genes. Based on the increased D-pantothenate accumulation as a consequence of the *ilvA* deletion, a further flux increase was attempted by overexpressing the common genes required for L-valine and L-isoleucine synthesis (Fig. 1). For this purpose pKK5 encoding *ilvBNC* (2) was used, thus resulting in high-level acetohydroxy acid synthase and isomeroreductase activities. In addition the recently cloned *ilvD* gene (30a) was used. This gene was ligated with pKK5 to yield pJC1*ilvBNCD*. As a further construct pECM3*ilvBNCD* was made, which confers chloramphenicol resistance in contrast to pJC1*ilvBNCD*. The plasmids were used to transform *C. glutamicum* and its *ilvA* deletion mutant. The strains constructed were cultivated in minimal medium, and L-valine accumulations were determined after 48 h of cultivation when glucose was consumed. The highest L-valine concentration obtained was 79 mM, whereas the wild type accumulated only 1 mM (Fig. 5). The strains with the *ilvA* deletions accumulated higher L-valine concentrations than the *ilvA*$^+$ strains. Furthermore, *ilvD* overexpression is necessary to obtain the maximal L-valine accumulation. As a third outcome, it is evident that the basis vector itself influences L-valine accumulations, although both vectors use the same *C. glutamicum* replicon.

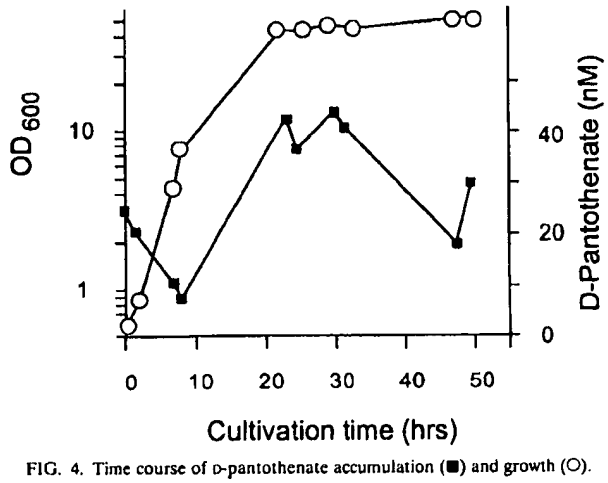

FIG. 4. Time course of D-pantothenate accumulation (■) and growth (○).

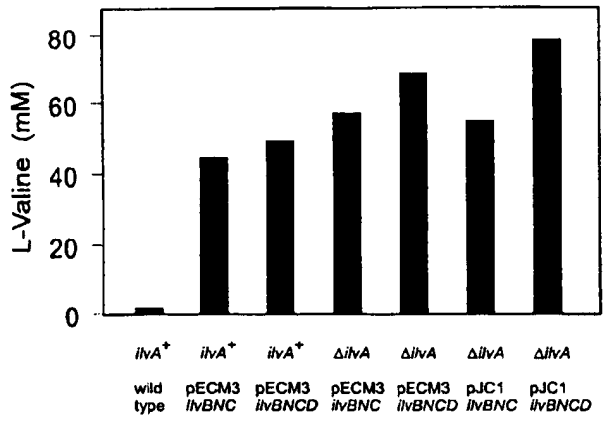

FIG. 5. L-Valine accumulation with isogenic *C. glutamicum* strains. Below the columns the genotype of each strain is given, which is either *ilvA*$^+$ or Δ*ilvA*. The strains additionally carry the plasmid pECM3 or pJC1 carrying *ilvBNC* or *ilvBNCD*, respectively.

D-Pantothenate accumulation by combined overexpression of *ilv* and *pan* genes. To exploit the increased capability of L-valine formation for increased D-pantothenate accumulation, the four *ilv* genes were overexpressed together with *panBC*. To enable their common overexpression, compatible plasmids were required. For this purpose, the chromosomal 2.2-kb fragment encompassing *panBC* was engineered, as outlined in Materials and Methods, to be cloned into the expression vector pEKEx2, which carries the pBL1 replicon (37) and confers kanamycin resistance, to yield pEKEx2*panBC*. Furthermore, *panBC* was ligated with pEC7 by using the same replicon (but conferring chloramphenicol resistance) to yield pEC7 *panBC*. Starting from the *C. glutamicum ilvA* deletion mutant carrying pJC1*ilvBNCD*, a *C. glutamicum ilvA* deletion mutant carrying pJC1*ilvBNCD* and pEC7*panBC* was made, and starting from the *C. glutamicum ilvA* deletion mutant carrying pECM3*ilvBNCD*, a *C. glutamicum ilvA* deletion mutant carrying pECM3*ilvBNCD* and pEKEx2*panBC* was made. The D-pantothenate accumulations obtained with these strains in the standard minimal medium containing 20 mM β-alanine are shown in Fig. 6. First of all, the L-valine-producing *C. glutamicum ilvA* deletion mutant carrying pJC1*ilvBNCD* and pEC7 (control [without *panBC* overexpressed]) already accumulated up to 0.53 mM D-pantothenate after 49 h (Fig. 6). Without β-alanine addition the accumulation was only 0.87 μM, thus showing the absolute requirement of this amino acid derivative for increased D-pantothenate formation (data not shown). When additionally *panBC* was overexpressed (the *ilvA* deletion mutant carrying pJC1*ilvBNCD* and pEC7*panBC*) the D-pantothenate was accumulated to a concentration of 2.1 mM. This strong effect of *panBC* overexpression is also apparent with the second gene combination. Whereas the *ilvA* deletion mutant carrying pECM3*ilvBNCD* and pEKEx2 accumulated 0.43 mM D-pantothenate, the *ilvA* deletion mutant carrying pEKEx2 *panBC* accumulated as much as a 4.2 mM concentration of the vitamin, which is a 10^5-fold-higher concentration than that obtained with the wild type. After 74 h the pantothenate accumulations quantified were almost the same as those at the earlier point in time.

DISCUSSION

In the present work the genes *panB* and *panC* of *C. glutamicum* were cloned and were found to be clustered. In *B. subtilis* and *E. coli* an identical organization of the two genes is present (28, 44). For the latter organism a transcriptional analysis has revealed a significantly larger transcript of *panB* than expected from the size of that gene, suggesting the cotranscription of a second gene (17). According to recent genome information this could well be *panC*. There is evidence that in *C. glutamicum panB* and *panC* constitute an operon. The sequence shows that both genes overlap by one nucleotide (Fig. 3), which has been demonstrated for amino acid biosynthetic genes to be evidence of a close translational coupling (31). In addition to this structural feature, the functional characterization of pantothenate synthetase activities supports the conclusion that *panBC* in *C. glutamicum* forms an operon. Whereas with a *panBC*-containing fragment an increased synthetase activity was the result, this was not the case with a fragment containing *panC*, which included a significant chromosomal part of the 5′ region of the gene.

The three enzymes of the D-pantothenate synthesis quantified have specific activities of around 1 nmol/min/mg of protein. This is extremely low compared to the specific activities of enzymes of amino acid synthesis, which are about 2 orders of magnitude higher, or that of enzymes of the central metabolism, whose specific activities are increased by as many as 3 orders of magnitude. This may be due to different enzyme amounts and consequently different expression levels. As the expression levels of genes of amino acid synthesis and of the central metabolism are shown to be directly related to the degree of codon bias in *C. glutamicum* (10), as is the case for other organisms too, it was interesting to inspect the codon usage of the cloned *pan* genes. This was done together with the biotin biosynthesis genes (*bioABD*) of *C. glutamicum* (13). This analysis revealed that in fact the codon usage of the vitamin biosynthesis genes of *C. glutamicum* is less biased than that of the high and moderately expressed genes. As a consequence, the preferred codon for vitamin biosynthesis genes is, in 6 of 19 cases, different from that of the high and moderately expressed genes, for which almost exclusively the same codon is used (data not shown). Since the D-pantothenate accumulation is in part dependent on the vector used (Fig. 6), which might reflect different expression levels, the design of *pan* genes by the use of appropriate codons is an option to obtain optimal expression levels for increased product accumulation.

From the enzyme activity determinations it is furthermore

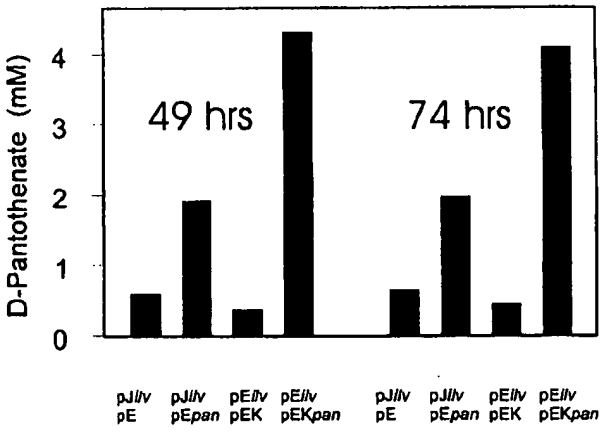

FIG. 6. D-Pantothenate accumulation with plasmid-carrying strains derived from the *C. glutamicum ilvA* deletion mutant. p*Jilv*, pJC1*ilvBNCD*; p*Eilv*, pECM 3*ilvBNCD*; p*Epan*, pEC7*panBC*; pEK*pan*, pEKEx2*panBC*; pE, pEC7.

evident that the pathway of D-pantothenate synthesis in the gram-positive bacterium *C. glutamicum* is identical to that of the gram-negative bacterium *E. coli*, where β-alanine is not uracil derived as in plants, for instance. Also the feedback inhibition of the ketopantoatehydroxymethyl transferase by pantoate is comparable in both organisms (33). The inhibition of the transferase activity of *C. glutamicum* by the false feedback inhibitor salicylate reflects the situation described for *S. typhimurium* (34). An important difference is the inhibition of the transferase in *E. coli* by D-pantothenate. In a concentration of 2.5 mM this effector reduces the enzyme activity of *E. coli* by about 50% (33), whereas the enzyme of *C. glutamicum* is almost unaffected by 10 mM D-pantothenate.

The increased D-pantothenate accumulation by *C. glutamicum* required a concerted engineering of the metabolite flux similar to that experienced during the construction of L-isoleucine-producing strains (7). One important feature in obtaining a D-pantothenate accumulation is the deletion of *ilvA*, which encodes the key enzyme of isoleucine synthesis (30). There are three possibilities of explaining this effect. The first is that the catalytic activity of the single acetohydroxy acid synthase present in *C. glutamicum* (20) is, after the deletion of *ilvA*, exclusively available for ketoisovalerate synthesis. The second is that L-isoleucine no longer exerts its inhibitory effect by an allosteric interaction with the acetohydroxy acid synthase (6). The third is that due to the introduced growth limitations, increased precursor metabolite concentrations are available to enter the biosynthesis pathway. This is known from several examples. For instance, a molecularly introduced growth limitation results in an increased L-lysine accumulation by *C. glutamicum* (8, 32), and a growth limitation obtained by an appropriate process management results in an increased L-phenylalanine accumulation by *E. coli* (21).

The successful use of *ilvBNCD* overexpression to obtain an increased D-pantothenate accumulation is due to the increased ketoisovalerate availability. Only then does the *panBC* overexpression result in a substantial accumulation of D-pantothenate. It therefore follows that an increased ketoisovalerate availability is mandatory to direct the metabolite flux into the D-pantothenate-specific part of the pathway with its low specific activities. Furthermore, the availability of β-alanine is essential, since without its addition no substantial amounts of D-pantothenate accumulate with the strain constructed. By using the appropriate tools and procedures developed in this study the low concentration of 10 μg of D-pantothenate per liter accumulated by the wild type of *C. glutamicum* was increased to the high concentration of about 1 g of the vitamin per liter. A further improvement of *C. glutamicum* appears possible to reach concentrations which are in the range of those obtained for the amino acids produced with this organism.

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